

ORIGINAL CONTRIBUTION

Case-Control Study of the *Parkin* Gene in Early-Onset Parkinson Disease

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Background: Mutations in *parkin* are estimated to account for as much as 50% of familial Parkinson disease (PD) and 18% of sporadic PD. Single heterozygous mutations in *parkin* in both familial and sporadic cases may also increase susceptibility to PD. To our knowledge, all previous studies have been restricted to PD cases; this is the first study to systematically screen the *parkin* coding regions and exon deletions and duplications in controls.

Objective: To determine the frequency and spectrum of *parkin* variants in early-onset PD cases (aged ≤ 50 years) and controls participating in a familial aggregation study.

Patients and Methods: We sequenced the *parkin* gene in 101 cases and 105 controls. All cases and controls were also screened for exon deletions and duplications by semi-quantitative multiplex polymerase chain reaction.

Results: Thirteen (12.9% [95% confidence interval, 7%-21%]) of the 101 cases had a previously described

parkin mutation: 1 was homozygous, 11 were heterozygous, and 1 was a compound heterozygote. The mutations Arg42Pro (exon 2) and Arg275Trp (exon 7) were recurrent. The previously reported synonymous substitution Leu261Leu (c.884A>G) was identified in 4 (3.9%) of 101 cases and 2 (2%) of 105 controls ($P=.44$). Excluding the synonymous substitution Leu261Leu (heterozygotes), 10 (9.9% [95% confidence interval, 4.6%-17.5%]) carried mutations.

Conclusions: The frequency of mutations among cases that were not selected based on family history of PD is similar to what has previously been reported in sporadic PD. The similar frequency of Leu261Leu in cases and controls suggests it is a normal variant rather than a disease-associated mutation. We confirmed that heterozygous *parkin* mutations may increase susceptibility for early-onset PD.

Arch Neurol. 2006;63:548-552

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MUTATIONS IN 4 GENES, *α -synuclein* (*PARK1*),^{1,2} *parkin* (*PARK2*),³ *DJ-1* (*PARK7*),^{4,6} and *PINK1* (*PARK6*),⁷⁻¹¹ are associated with early-onset Parkinson disease (EOPD). *Parkin* mutations account for the majority of familial and sporadic EOPD cases with a known genetic association.^{3,12} In PD cases with age at onset (AAO) of 45 years or younger, the frequency of *parkin* mutations is estimated at 49% in cases with a family history of PD¹³ and 15% in cases without a family history of PD.¹⁴ In the only community-based study, the frequency of mutations was 9% in 111 cases with AAO of 50 years or younger.¹⁵

In both familial and sporadic cases, homozygous, compound heterozygous, and single heterozygous mutations in *parkin* have been described. Several studies indicate that heterozygous mutations

may increase susceptibility to PD,^{3,16-19} although this is controversial. The carrier (heterozygous) frequency of *parkin* alleles in the normal population is unknown. Previous studies have only assessed the frequency of specific *parkin* mutations and/or variants that were identified in PD cases and have not systematically screened the *parkin* gene for mutations in normal controls.^{3,20}

We have screened participants in the Genetic Epidemiology of PD Study (GEPD) for variants in *DJ-1*²¹ and *glucocerebrosidase*.²² In the present study, to identify variants in the *parkin* gene, coding exons were sequenced completely in 206 subjects, including 101 EOPD cases and 105 controls from GEPD. All subjects (cases and controls) were also screened for exon deletions and duplications by semi-quantitative multiplex polymerase chain reaction (PCR).

SUBJECTS

Cases

Cases were recruited based on AAO of motor signs of 50 years or younger (EOPD), regardless of family history of PD in a first-degree relative. All cases were seen at the Center for Parkinson's Disease and Other Movement Disorders at Columbia University, and EOPD cases were oversampled.²³ Duration of PD was calculated as the years from motor onset to enrollment in GEPD. We have previously shown that reliability of reporting the AAO of motor signs of PD was excellent.²³ All *parkin* coding exons were sequenced and analysis of exon deletions and duplications were completed in 101 of 256 EOPD cases from GEPD²³ on whom data were complete at the time of the analysis. The current study includes 21 of 33 cases previously screened for *parkin* variants.²⁴ Analysis was performed without knowledge of these results.

Controls

One hundred five of 412 controls from GEPD on whom data were complete were randomly chosen for sequencing of *parkin* exons and analysis of exon deletions and duplications by semiquantitative multiplex PCR. For PD cases recruited from the Center for Parkinson's Disease and Other Movement Disorders, controls were recruited by random-digit dialing and were frequency matched based on key demographic variables.²³ For cases who were residents of Washington Heights, Manhattan, NY, and 65 years or older, controls were recruited from a 50% sample of names and addresses of Medicare recipients provided by Health Care Finance Association. For 18 cases younger than 65 years, controls were recruited from the Northern Manhattan Stroke Study in Washington Heights.²⁵ For the analysis of variants in exon 7, including the synonymous substitution Leu261Leu, an additional 82 Hispanic controls from the 50% Medicare sample were included to enrich the number of Hispanic controls.

All PD case and control probands underwent an evaluation that included a medical history and Unified Parkinson's Disease Rating Scale²⁶ and videotape assessment. The modified Mini-Mental State Examination was administered to all probands in either English or Spanish.²⁷ A valid, reliable, structured family history of PD questionnaire (Family History Information) was administered in English or Spanish to all PD cases and controls, either in person or over the telephone, for ascertainment of PD and other neurologic disease in first-degree relatives.²⁸ The institutional review board at the College of Physicians and Surgeons, Columbia University, approved this study. Informed consent was obtained from all study participants.

MOLECULAR GENETIC ANALYSIS

DNA was isolated from whole blood using standard techniques. All blood and genomic DNA samples were coded on receipt to ensure patient confidentiality. Polymerase chain reaction amplification of *parkin* exons 1 to 12 was performed in cases and controls. The primers used for PCR amplification of *parkin* exons and intronic and exonic boundaries and sequencing have been described previously.^{29,30} Cycle sequencing was performed on the purified PCR product as per the manufacturer's instructions (BigDye; Applied Biosystems, Foster City, Calif). Products were analyzed on an ABI3700 genetic analyzer (Applied Biosystems). Chromatograms were viewed using Sequencher (Gene Codes Corporation, Ann Arbor, Mich) and sequence variants determined. All sequence variants identified

in cases and controls were confirmed by analysis in a separate PCR followed by bidirectional sequencing. Sequence variants were classified as polymorphic if their frequency was 1% or greater in ethnically matched controls. These analyses were performed without knowledge of case-control status.

To identify genomic deletions and exon rearrangements in *parkin*, semiquantitative multiplex PCR was performed as previously described.³¹ Briefly, Hex-tagged fluorescent-labeled primers for *parkin* exons were optimized in pooled sets of 4 primer pairs for multiplexing along with an internal control (328-bp PCR product of an unlinked gene transthyretin on chromosome 18). The PCR fragments were analyzed on an ABI3700 genetic analyzer. Electropherograms were viewed and product size and peak heights calculated using Genescan 3.1 and Genotyper software (Applied Biosystems).

DATA ANALYSIS

Baseline characteristics at the time of enrollment were compared using the *t* test for continuous variables and the χ^2 test or Fisher exact test for categorical variables. First, we compared the demographic characteristics of the subjects who were included in the analysis of *parkin* variants with those of the subjects who were not. Second, we compared the demographic characteristics of cases and controls who were analyzed for *parkin*. Third, we compared the demographic and clinical characteristics of cases with and without mutations in the *parkin* gene. The Wilcoxon rank sum test was used to compare the total modified Mini-Mental State Examination score in PD cases with and without *parkin* mutations because of the inadequacy of normality assumption of the distribution.

In a separate analysis including an additional 82 Hispanic controls, we compared the frequency of Leu261Leu in cases and controls and performed a stratified analysis by ethnic group to determine whether there was an association with disease status.

RESULTS

DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF THE CASE-CONTROL COHORT

At the time of this study, 668 subjects including 256 EOPD cases and 412 controls had been recruited into GEPD. All *parkin* coding exons were sequenced and analyzed for exon deletions and duplications in a subset of 206 (101 PD cases with AAO \leq 50 years and 105 controls). Demographic and clinical characteristics of the 206 cases and controls are presented in **Table 1**. The mean (SD) AAO of 101 cases was 41.1 (7.2) years, disease duration was 11.7 (8.0) years, and total motor score (Unified Parkinson's Disease Rating Scale Part III) was 21.0 (13.00). Compared with the subjects in whom *parkin* was not sequenced (n=462), the 206 cases and controls were younger (56.9 vs 63.2 years; $P < .001$), had completed more years of education (15.6 vs 14.5 years; $P < .001$), and were more likely to be white (89.3% vs 66.8%; $P < .03$). Cases in whom the *parkin* gene was sequenced and analyzed for exon deletions and duplications (n=101) did not differ significantly from cases who were not analyzed (n=155) in AAO of PD, age at evaluation, years of education, ethnicity, sex, or family history of PD. Those cases in whom the gene was analyzed had longer duration of PD (11.9 vs 10.0 years; $P = .05$); however, Hoehn and Yahr score and total motor score on the Unified Parkinson's Disease Rating Scale Part III were similar to nonsequenced cases.

Table 1. Demographics of Analyzed Cases and Controls

| | Total (N = 206) | Cases (n = 101) | Controls (n = 105) | P Value, Cases vs Controls |
|--------------------------------------|--------------------|--------------------|-----------------------|-------------------------------|
| Male, No. (%) | 119 (57.8) | 60 (59.4) | 59 (56.2) | .64 |
| Age, y, mean (SD) | 56.9 (10.9) | 53.0 (9.8) | 60.6 (10.6) | .001 |
| Ethnicity, No. (%) | | | | |
| White | 184 (89.3) | 87 (86.1) | 97 (92.4) | .04* |
| African American | 3 (1.5) | 0 | 3 (2.9) | |
| Hispanic | 15 (7.3) | 11 (10.9) | 4 (3.8) | |
| Other | 4 (1.9) | 3 (3.0) | 1 (1.0) | |
| Education, y, mean (SD) | 15.6 (3.0) | 15.7 (3.1) | 15.5 (2.9) | .61 |
| With <i>parkin</i> variants, No. (%) | 11 (5.34) | 11 (10.9) | 0 | <.001 |
| With family history of PD, No. (%)† | 19 (9.4) | 13 (13.4) | 6 (5.7) | .06 |

Abbreviation: PD, Parkinson disease.

*Fisher exact test.

†Family history was available for 202 subjects (97 cases and 105 controls).

Table 2. *Parkin* Mutations Identified in Cases

| Patient | Exon | Mutation | Zygoty | Ethnicity | Age at Onset, y |
|---------|------|-----------------------------------|-----------------------|--------------------|-----------------|
| 1 | 1 | c.81G>T and Leu261Leu | Compound heterozygous | Hispanic | 28 |
| 2 | 2 | Arg42Pro | Heterozygous | White non-Hispanic | 44 |
| 3 | 2 | Arg42Pro | Heterozygous | White non-Hispanic | 44 |
| 4 | 3 | Exon 3 40-bp deletion (c.438-477) | Homozygous | White non-Hispanic | 38 |
| 5 | 3, 4 | Exons 3 and 4 deletion | Heterozygous | Hispanic | 16 |
| 6 | 5 | Met192Leu | Heterozygous | Hispanic | 36 |
| 7 | 5 | Exon 5 deletion | Heterozygous | White non-Hispanic | 48 |
| 8 | 5 | Exon 5 deletion | Heterozygous | White non-Hispanic | 50 |
| 9 | 7 | Leu261Leu | Heterozygous | White non-Hispanic | 41 |
| 10 | 7 | Leu261Leu | Heterozygous | Hispanic | 41 |
| 11 | 7 | Leu261Leu | Heterozygous | Hispanic | 47 |
| 12 | 7 | Arg275Trp | Heterozygous | White non-Hispanic | 47 |
| 13 | 7 | Arg275Trp | Heterozygous | Other | 24 |

FREQUENCY OF PARKIN MUTATIONS IN CASES AND CONTROLS

Thirteen (12.9%) (95% confidence interval [CI], 7%-21%) of the 101 cases had a previously described *parkin* mutation; 1 was homozygous, 11 were heterozygous, and 1 was a compound heterozygote (**Table 2**). Five different point mutations and 3 different exon rearrangements were identified (Table 2). Point mutations included 3 missense mutations, 1 synonymous substitution, and 1 splice mutation. Exon deletions were found in 3 different exons (exons 3, 4, and 5). Fifty-four percent (7/13) of the variants identified in cases were found in exons encoding functional domains including the ubiquitin domain (exons 2 and 3) and RING1 domain (exon 7). The mutations Arg275Trp, Arg42Pro, and the synonymous substitution Leu261Leu were recurrent; 2 cases carried Arg275Trp, 2 carried Arg42Pro, and 4, including 1 compound heterozygote, carried Leu261Leu (3 Hispanic subjects, 1 white non-Hispanic subject). The synonymous substitution Leu261Leu was identified in 2% (2/105) of controls (1 Hispanic subject and 1 African American subject). To our knowledge, all previously published studies have evaluated the Leu261Leu allele frequency only in white non-Hispanic control subjects. Because we identified the syn-

onymous substitution in Hispanic and white non-Hispanic subjects and our pool of Hispanic controls was limited (n=4), we added 82 Hispanic controls from the 50% Health Care Finance Association sample to the existing control group (n=105). We found that 13% (11/86) of Hispanic controls carried the synonymous substitution Leu261Leu (**Table 3**). The allele frequency was higher in Hispanic cases and controls combined, 14.4% (14/97), compared with all other ethnicities, including white non-Hispanic, African American, and Asian cases and controls combined (1.1% [2/191]) ($P<.001$).

FREQUENCY OF PARKIN POLYMORPHISMS

The previously reported *parkin* polymorphisms, IVS3-20C>T, IVS2 + 25T>C, IVS7-35A>G, Ser167Asn, Asp394Asn, and Val380Leu, were observed at a similar frequency in cases and controls and were not significantly associated with PD (data not shown).

CLINICAL CHARACTERISTICS OF CASES WITH AND WITHOUT PARKIN MUTATIONS

Demographic and clinical characteristics of 91 cases without *parkin* mutations and 10 cases (excluding 3 cases with

Table 3. Leu261Leu Synonymous Substitution Status and Case-Control Status Stratified by Ethnicity in 101 Cases and 187 Controls

| Ethnicity | Carrier Sample Size | Noncarrier Sample Size | Total | P Value |
|-----------|---------------------|------------------------|-------|---------|
| White | | | | |
| Case | 1 | 86 | 87 | .47 |
| Control | 0 | 97 | 97 | |
| Total | 1 | 183 | 184 | |
| Hispanic | | | | |
| Case | 3 | 8 | 11 | .19 |
| Control | 11 | 75 | 86 | |
| Total | 14 | 83 | 97 | |
| Other | | | | |
| Case | 0 | 3 | 3 | >.99 |
| Control | 1 | 3 | 4 | |
| Total | 1 | 6 | 7 | |
| Total | | | | |
| Case | 4 | 97 | 101 | .44 |
| Control | 12 | 175 | 187 | |
| Total | 16 | 272 | 288 | |

the synonymous substitution Leu261Leu) with mutations are presented in **Table 4**. Information on history of PD in first-degree relatives was available for 97 of 101 cases and 9 of 11 *parkin* mutation carriers. The frequency of *parkin* mutations was 15.4% (2/13) in cases with a family history of PD in a first-degree relative, compared with 8.3% (7/84) in those without a family history of PD ($P=.35$). The 2 cases with *parkin* mutations who reported a family history of PD in a first-degree relative had AAOs of 32 and 38 years, respectively. One of the probands, the only homozygote (AAO, 38 years), had 2 siblings with PD (AAOs, 26 and 30 years). The frequency of *parkin* mutations ($n=10$) was 50% (1/2) in cases with AAO of 20 years and younger, 33% (2/6) in cases with AAO of 21 to 30 years, 9% (3/33) in cases with AAO of 31 to 40 years, and 7% (4/60) in cases with AAO of 41 to 50 years. Noncarriers and carriers did not differ in terms of first symptom reported (rest tremor, bradykinesia, rigidity, or gait impairment) nor did they differ in terms of current use of levodopa/carbidopa or dopamine agonists. None of the cases reported hallucinations or cognitive impairment preceding motor signs. Cases were not specifically queried about sleep benefit or sensitivity to levodopa in GEPD.

COMMENT

The prevalence of *parkin* mutations in 101 cases with AAO younger than 50 years was 9.9% (95% CI, 4.9%-17.5%) (excluding 3 heterozygous cases with the synonymous substitution Leu261Leu) and 8.3% (95% CI, 3.4%-16.4%) among those cases who did not report a family history of PD in a first-degree relative, which is within the range (9%-18%) reported in other series comprising primarily sporadic cases.^{14,15,32} The frequency of *parkin* mutations in cases with AAO younger than 45 years in our study was 11.1% (95% CI, 4.6%-21.6%), which is very similar to the 15% frequency reported by Periquet et al¹⁴ in pa-

Table 4. Cases With and Without *Parkin* Mutations Excluding 3 With Leu261Leu Mutation

| | No Mutation (n = 90) | Mutation (n = 11) | P Value |
|--|----------------------|-------------------|---------|
| Current age, y, mean (SD) | 53.8 (9.7) | 45.8 (8.0) | .01 |
| Age at onset, y, mean (SD) | 41.6 (6.5) | 36.3 (11.3) | .18 |
| Male, No. (%) | 57 (62.6) | 3 (30.0) | .08 |
| Ethnicity, No. (%) | | | .05 |
| White | 81 (89.0) | 6 (60.0) | |
| Hispanic | 8 (8.8) | 3 (30.0) | |
| Other | 2 (2.2) | 1 (10.0) | |
| Education, y, mean (SD) | 15.9 (3.1) | 13.6 (2.6) | .02 |
| Duration of PD, y, mean (SD) | 12.2 (8.2) | 9.5 (6.4) | .24 |
| UPDRS Part III ²⁶ total | 21.4 (13.4) | 17.5 (9.4) | .26 |
| motor score, mean (SD) | | | |
| mMMSE ²⁷ total score (SD) | 53.7 (4.5) | 53.2 (5.4) | .72 |
| Family history of PD in first-degree relative, % (No./Total No.) | 12.5 (11/88) | 22.2 (2/9) | .35 |
| Bradykinesia, % | 78.0 | 90.0 | .68 |
| Rest tremor, % | 77.8 | 70.0 | .69 |
| Rigidity, % | 98.9 | 100.0 | >.99 |
| Postural instability, % | 50.6 | 20.0 | .09 |
| Asymmetry, % | 83.2 | 100.0 | .35 |
| Improvement with levodopa, % | 98.5 | 100.0 | >.99 |
| Taking levodopa, % | 76.4 | 66.7 | .68 |
| Presence of dyskinesias, % | 47.1 | 50.0 | >.99 |
| Hoehn and Yahr score, mean (SD) | 2.3 (0.97) | 2.1 (0.17) | .08 |

Abbreviations: mMMSE, modified Mini-Mental State Examination; PD, Parkinson disease; UPDRS, Unified Parkinson's Disease Rating Scale.

tients with isolated early-onset parkinsonism (AAO, <45 years). Phenotypic differences were not observed between cases with and without *parkin* mutations in this study; however, detailed psychiatric and cognitive assessments were not performed. The synonymous substitution Leu261Leu (c.884A>G), which has been previously described as a mutation,^{24,33} was identified with similar frequency in cases and controls, suggesting that it is a common variant rather than a disease-associated mutation. The frequency of this variant appeared to be higher in Hispanic subjects than in non-Hispanic subjects. Our current sample size was insufficient to assess definitively the possibility that the synonymous substitution Leu261Leu is a susceptibility allele, and functional data are not available to determine pathogenicity. Based on the allele frequencies we observed in subjects of Hispanic ethnicity, a sample size of 934 (467 cases and 467 controls) would have been required to detect a statistically significant difference (80% power, $\alpha=.05$).

To our knowledge, this is the first study to describe complete sequencing of *parkin* coding exons and analysis of exon deletions and duplications by semiquantitative PCR in cases and controls. Several studies have reported heterozygous *parkin* mutations in both EOPD and late-onset PD.^{18,20,24,29,32-40} Currently, the identification of heterozygous mutations in patients with PD is controversial, and it is unknown whether heterozygous mutations alone are pathogenic or whether additional mutations have been missed in screening the *parkin* gene in these studies. Our current study helps clarify this issue by screening controls and finding that heterozygous mutations are absent. The

absence of control subjects carrying *parkin* mutations suggests that heterozygous *parkin* mutations may increase susceptibility for EOPD. There are few functional studies to support the hypothesis that *parkin* heterozygous mutations are pathogenic. However, 1 study⁴¹ has demonstrated that 2 RING finger 1 mutations (R256C and R275W) are dominant gain-of-function mutations, which is consistent with the identification of heterozygous mutations in several studies and supports the hypothesis that *parkin* heterozygous mutations are pathogenic. Other strengths of this study include that all cases and controls from GEPD were administered reliable, structured neurologic examinations and family history of PD interviews. The identification of possible ethnic differences in allele frequencies for the Leu261Leu variant highlights the importance of well-characterized multiethnic samples in determining disease susceptibility alleles and in diagnostic screening.

Accepted for Publication: October 12, 2005.

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Funding/Support: This study was supported by grants by NS36630 (Dr Marder), RR00645, AG07232, NS39422, and NS29993 from the National Institutes of Health and the Parkinson's Disease Foundation (Dr Clark).

Acknowledgement: We appreciate the assistance of Richard Mayeux, MD, MSc, Ralph Sacco, MD, and Paul Greene, MD, for the recruitment of patients. We also thank Christine Klein, MD, and Katja Hedrich, PhD.

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